AD_____

Award Number: DAMD17-99-1-9095

TITLE: DNA Methylation Alterations in Breast Cancer

PRINCIPAL INVESTIGATOR: Fumiichiro Yamamoto, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020913 054

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (01 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
DNA Methylation Alterations in Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9095

6. AUTHOR(S)
Fumiichiro Yamamoto, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Burnham Institute
La Jolla, California 92037

E-Mail: fyamamoto@burnham.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

We have characterized one band that exhibited a conspicuous increase in band intensity in breast cancer *NotI-MseI* MS-AFLP DNA fingerprints. The band contained a satellite DNA sequence in the pericentromeric regions of chromosomes. We have shown that this intensity change is frequently observed in breast cancer tissues and breast carcinoma cell lines and that it is caused by hypomethylation.

14. SUBJECT TERMS
Breast cancer, DNA methylation, genome scanning, genomics, MS-AFLP, DNA fingerprinting

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The deoxycytosine methylation of CpG dinucleotides is one of the most common modifications of DNA, and the molecular mechanisms regulating these modulations (the loss of the methylated status, i.e., hypomethylation, and the methylation of a previously unmethylated nucleotide, i.e., hypermethylation) has been gradually elucidated. A hypothesis currently accepted is that these subtle alterations in DNA structure may change the chromatin structure, which occasionally result in a fine-tuning of gene expression (1). In addition to normal physiological roles in such fundamental processes as embryogenesis, cell differentiation and aging (2-5), DNA methylation alterations have also been shown to play an important role in pathological processes, one of which is exemplified by cancer development and progression (6,7).

Hypermethylation of CpG islands in the promoter region has been closely correlated with the silencing of transcription of certain genes during tumorigenesis (8-11). Therefore, we thought that it might be possible to identify the genes involved in tumorigenesis by scanning the alterations in DNA methylation. For this purpose, we developed a technique, called methylation sensitive-amplified fragment length polymorphism (MS-AFLP). MS-AFLP is an efficient and sensitive method that permits the evaluation of the spectrum of genetic and epigenetic alterations on a genomic scale. The technique utilizes the DNA fingerprinting advantages of the AFLP (12) and methylation sensitivity of the *NotI* restriction enzyme. The total number of *NotI* sites is estimated to be 100,000, and about 5,300 distinct sites, excluding repeats, are unmethylated and cleavable in certain tissues (13).

During the previous year 1, we performed the MS-AFLP DNA fingerprinting experiments for scanning alterations in tumor DNA, using 10 matched normal and tumor pairs of DNA samples from breast cancer patients, and identified many bands that exhibited changes in band intensity. We cut out the dried gel slices from those bands, eluted, PCR-amplified, and cloned DNA fragments into pT-Adv plasmid vector by the T-A cloning method. After transformation of *E. coli* bacteria, plasmid DNA was prepared from several transformant clones, and the nucleotide sequences of the inserts were determined. For some bands, we also transferred DNA from the DNA fingerprints onto nylon membranes by electrotransfer, hybridized those membranes with the probes prepared from individual insert fragments from the sequence-verified clones, and determined the identity of the fragments showing alterations. We identified a high number of entries related to the homeobox genes and genes involved in the regulation of homeotic gene expression. Because regulated cell growth and differentiation are the basis of development, and cancer results from uncontrolled growth of undifferentiated cells, it is not difficult to speculate that activation/inactivation of certain homeotic genes may contribute to carcinogenesis. Therefore, our results seem to have demonstrated the capability of the *NotI-MseI* MS-AFLP method to identify and isolate the genes whose changes may be involved in cancer development and/or progression.

BODY

Proposed Task 1. To investigate breast cancer-associated DNA methylation changes (months 1-18).

1. *NotI-MseI* MS-AFLP will be performed with 10 sets of normal and tumor DNA from breast cancer patients
   4x4 format using combinations of four *NotI* and four *MseI* primers with an additional selective residue (G, A, T, or C) at the 3' end (months 1-6)
   4x16 format using four *NotI* with one additional residue and sixteen *MseI* with two additional selective residues (GG, GA, GT, GC, AG, AA, AT, AC, TG, TA, TT, TC, CG, CA, CT, or CC) (months 7-24)

The proposed task 1 was accomplished in the year 1.

Proposed Task 2. To determine the identity of bands exhibiting consistent changes in breast cancers (months 2-36).

1. Alterations in band intensity will be identified, and DNA fragments will be cloned from individual bands showing alterations (months 2-20)
2. Plasmid clones containing the inserts exhibiting the alterations will be identified (months 3-24)
3. Nucleotide sequences will be determined (months 4-28)
4. BLAST search will be performed to identify the identical/homologous sequences in the database (months 5-32)
5. Important fragments will be mapped on chromosome using GeneBridge 4 Radiation Hybrid panel (months 6-36)---This subtask has been cancelled because more than 97% of the human genome have already been sequenced and there is no need to experimentally determine the chromosomal loci.
6. 5'- and 3'-RACE will be performed to clone the entire cDNAs for the sequences showing high homology with those deposited in EST database but not identical to the known genes (months 13-36)
7. RT-PCR will be performed to examine the effects of DNA methylation alterations on gene expression (months 19-36)

In the year 1, alterations in band intensity were identified and DNA fragments were cloned from the bands exhibiting alterations (subtask 1), the nucleotide sequences of the cloned fragments determined (subtask 3), and BLAST searches conducted (subtask 4). For some bands, the clones containing the inserts that exhibited the alterations were identified, by hybridizing the MS-AFLP fingerprint electroblots with radiolabeled probes prepared from individual candidate plasmid inserts (subtask 2). We found that the confirmation procedures were time-consuming and costly because multiple hybridization experiments were usually necessary before identifying a right clone. In order to facilitate this confirmation process, we thought of developing an easier method that utilizes hybridization of dot-blotted cloned fragments with MS-AFLP fingerprint probes. However, the attempt has been unsuccessful (We are still working on this endeavor). For dozens of bands, therefore, real sequences that exhibit alterations remain to be determined. The last two subtasks (6 and 7) will be performed in the year 3 after the identities of the additional bands have been determined.

We characterized the band in one of the *NotI-MseI* MS-AFLP fingerprints, which exhibited a conspicuous increase in band intensity in the DNA fingerprint of the metastasized breast tumor. A homology search revealed that the sequence had significant homology with tandemly repeated sequences from pericentromeres of chromosomes 9, 13, 14, and 21 (U59100: 5e-69 probability and Y10572: 7e-65). Genomic DNA from additional breast cancer cases was next examined for this alteration by the *NotI-MseI* MS-AFLP. Stronger band intensity in tumor was observed in a majority of breast cancer patients (12 out of 17 cases examined). Genomic DNA from breast cancer cell lines was also examined for the alteration. Strong signal was observed with DNA from Hs 578T cell line and weak signal was observed with DNA from MDA-MB468 and BT-20 cell lines. DNA from these fingerprints was electrotransferred onto a nylon membrane and hybridized with the radiolabeled cloned satellite DNA fragment probe. The identical banding pattern was obtained, confirming the identity of the band. Weak signal was detected with DNA from CAL-51 cells by this method with higher sensitivity. No signal was observed with DNA from MCF-7.

We then performed the Southern hybridization experiment. Genomic DNA from MCF-7, MDA-MB468, and Hs 578T cells was digested with *NotI* or *HpaII*, gel electrophoresed, and Southern transferred. The relative hybridization signal to DNA amount seemed to be stronger with MDA-MB468 DNA than those with MCF-7 and Hs 578T DNA. This may be due to a difference in the degree of homology to the nucleotide sequence of the repeat fragment used as a probe or a difference in the number of repeats. Nonetheless, differential susceptibility to those methylation sensitive restriction enzymes was obvious. The results also showed that the neighboring *HpaII* sites were also hypomethylated when the *NotI* site was hypomethylated.

The centromere is the constricted region of a chromosome at which the chromosome is attached to microtubules, and therefore, is essential for chromosomal segregation during mitosis. The pericentromeres, the regions flanking the centromere are often rich in short repeated satellite DNA sequences and also contain a considerable amount of constitutive heterochromatin. A deficiency in genomic methylation at satellite DNA sequences on either side of the centromeres has been reported in the ICF syndrome (immunodeficiency, centromeric region instability, facial anomalies) (14-16). Mutations have recently been found in the coding sequence of Dnmt3b DNA methyltransferase gene in the patients with this hereditary disorder (17-19). In addition to this syndrome, hypomethylation in the satellite DNA sequences of the pericentromeric regions of chromosomes has also been reported in various cancers. These include melanoma cell lines (20), immunodeficiency virus-related non-Hodgkin's lymphoma (21), breast adenocarcinomas (22), ovarian cancers (23), and Wilms tumors (24). We found that the *NotI* site in the tandem repeat sequences from pericentromeres of chromosomes 9, 13, 14, and 21 were hypomethylated in a majority of breast tumors. Since the hypomethylation of this *NotI* site has been reported in neuroblastomas (25) and HBV-integrated hepatocellular carcinomas (26), this satellite hypomethylation may be important in carcinogenesis, predisposing cells to structural and numerical chromosomal aberrations through the altered interaction with centromere binding proteins (27).

KEY RESEARCH ACCOMPLISHMENTS

1. We have determined the identity of one band that exhibited an increase in band intensity in the breast tumor MS-AFLP fingerprints. The sequence was derived from a satellite DNA sequence of the pericentromeric regions of chromosomes. We have observed this alteration in a majority of breast cancer cases and in some of the established breast cancer cell lines but not in colon cancer cases. Using the Southern hybridization, we have shown that the alteration resulted from hypomethylation rather than the (sub-)chromosomal loss of the sequence.

REPORTABLE OUTCOMES

1. The paper that described the technical details of *NotI-MseI* MS-AFLP technique was submitted, accepted, and published (F. Yamamoto, M. Yamamoto, J-L. Soto, E. Kojima, E.N. Wang, M. Perucho, T. Sekiya, and H. Yamanaka (2001). *NotI-MseI* Methylation Sensitive-Amplified Fragment Length Polymorphism (MS-AFLP) for DNA methylation analysis of human cancers. Electrophoresis 22: 1946-1956.

2. The identity of the band that exhibited hypomethylation in the MS-AFLP fingerprints of tumor DNA in a majority of breast cancer cases was determined to have derived from a satellite DNA sequence of the pericentromeric region of chromosomes. The detailed analysis of this band was also included in the above paper.

The satellite sequence is present in a large number of copies in the genome. Therefore, it seems likely that the detection of hypomethylation of this satellite sequence is much easier than the detection of hypermethylation of any single-copy genes. For this reason, we believe that detecting hypomethylation of satellite DNA sequence(s) using DNA prepared from sera is potentially useful for cancer diagnosis. (Note that hypomethylation of this sequence is not breast cancer-specific since it has also been observed in the tumors of neuroblastomas (25) and HBV-integrated hepatocellular carcinomas (26)).

CONCLUSIONS

We identified many changes in band intensity by scanning the *NotI-MseI* MS-AFLP DNA fingerprints between normal and tumor DNA from breast cancer patients. In the year 2, we characterized a band that exhibited a conspicuous increase in band intensity. The band is derived from the satellite DNA sequence in the pericentromeric region of chromosomes, and the utility of detecting hypomethylation of repeat sequence(s) in serum DNA for cancer diagnosis may be possible in the future.

REFERENCES

1. Jones, P.L. *et al.* Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* **19**, 187-191 (1998).

2. Issa, J.P. *et al.* Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat Genet* **7**, 536-540 (1994).

3. Counts, J.L. & Goodman, J.I. Alterations in DNA methylation may play a variety of roles in carcinogenesis. *Cell* **83**, 13-15 (1995).

4. Kass, S.U., Pruss, D. & Wolffe, A.P. How does DNA methylation repress transcription? *Trends Genet* **13**, 444-449 (1997).

5. Cedar, H. & Verdine, G.L. Gene expression. The amazing demethylase [news; comment]. *Nature* **397**, 568-569 (1999).

6. Baylin, S.B., Herman, J.G., Graff, J.R., Vertino, P.M. & Issa, J.P. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* **72**, 141-196 (1998).

7. Jones, P.A. & Laird, P.W. Cancer epigenetics comes of age. *Nat Genet* **21**, 163-167 (1999).

8. Laird, P.W. & Jaenisch, R. DNA methylation and cancer. *Hum Mol Genet* **3**, 1487-1495 (1994).

9. Graff, J.R. *et al.* E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* **55**, 5195-5199 (1995).

10. Klein, C.B. & Costa, M. DNA methylation, heterochromatin and epigenetic carcinogens. *Mutat Res* **386**, 163-180 (1997).

11. Kane, M.F. *et al.* Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res* **57**, 808-811 (1997).

12. Vos, P. *et al.* AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**, 4407-4414 (1995).

13. Yoshikawa, H. *et al.* Chromosomal assignment of human genomic NotI restriction fragments in a two-dimensional electrophoresis profile. *Genomics* **31**, 28-35 (1996).

14. Miniou, P. *et al.* Abnormal methylation pattern in constitutive and facultative (X inactive chromosome) heterochromatin of ICF patients. *Hum Mol Genet* **3**, 2093-2102 (1994).

15. Smeets, D.F. *et al.* ICF syndrome: a new case and review of the literature. *Hum Genet* **94**, 240-246 (1994).

16. Jeanpierre, M. *et al.* An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. *Hum Mol Genet* **2**, 731-735 (1993).

17. Hansen, R.S. *et al.* The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *Proc Natl Acad Sci U S A* **96**, 14412-14417 (1999).

18. Okano, M., Bell, D.W., Haber, D.A. & Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**, 247-257 (1999).

19. Chen, R.Z., Pettersson, U., Beard, C., Jackson-Grusby, L. & Jaenisch, R. DNA hypomethylation leads to elevated mutation rates. *Nature* **395**, 89-93 (1998).

20. Miwa, W., Yashima, K., Sekine, T. & Sekiya, T. Demethylation of a repetitive DNA sequence in human cancers. *Electrophoresis* **16**, 227-232 (1995).

21. Sawyer, J.R., Swanson, C.M., Koller, M.A., North, P.E. & Ross, S.W. Centromeric instability of chromosome 1 resulting in multibranched chromosomes, telomeric fusions, and "jumping translocations" of 1q in a human immunodeficiency virus-related non-Hodgkin's lymphoma. *Cancer* **76**, 1238-1244 (1995).

22. Narayan, A. *et al.* Hypomethylation of pericentromeric DNA in breast adenocarcinomas. *Int J Cancer* **77**, 833-838 (1998).

23. Qu, G., Dubeau, L., Narayan, A., Yu, M.C. & Ehrlich, M. Satellite DNA hypomethylation vs. overall genomic hypomethylation in ovarian epithelial tumors of different malignant potential. *Mutat Res* **423**, 91-101 (1999).

24. Qu, G.Z., Grundy, P.E., Narayan, A. & Ehrlich, M. Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. *Cancer Genet Cytogenet* **109**, 34-39 (1999).

25. Thoraval, D. *et al.* Demethylation of repetitive DNA sequences in neuroblastoma. *Genes Chromosomes Cancer* **17**, 234-244 (1996).

26. Nagai, H. *et al.* Isolation of NotI clusters hypomethylated in HBV-integrated hepatocellular carcinomas by two-dimensional electrophoresis. *DNA Res* **6**, 219-225 (1999).

27. Kipling, D. & Warburton, P.E. Centromeres, CENP-B and Tigger too. *Trends Genet* **13**, 141-145 (1997).

Electrophoresis 2001, *22*, 1946–1956

Fumiichiro Yamamoto[1]
Miyako Yamamoto[1]
Jose Luis Soto[1]
Eijiro Kojima[1]
Emily N. Wang[1]
Manuel Perucho[1]
Takao Sekiya[2]
Hiroyasu Yamanaka[3]

[1]The Burnham Institute, La Jolla Cancer Research Center, La Jolla, CA, USA
[2]Oncogene Division, National Cancer Center Research Institute, Tokyo, Japan
[3]Tokushima Bunri University, Tokushima, Japan

*Not*I-*Mse*I methylation-sensitive amplified fragment length polymorphism for DNA methylation analysis of human cancers

We have applied a methylation-sensitive restriction endonuclease, *Not*I, to the existing amplified fragment length polymorphism (AFLP) method and developed *Not*I-*Mse*I methylation-sensitive-AFLP (MS-AFLP). *Not*I-*Mse*I MS-AFLP allows the analysis of DNA methylation alterations at the *Not*I sites scattered over the genome. Hypermethylation and hypomethylation are visualized by the decrease and increase in the band intensity of DNA fingerprints. Identification of consistent changes can be facilitated through parallel electrophoresis of multiple samples. DNA fragments exhibiting alterations can be cloned from fingerprint bands by amplification of gel-eluted DNA with the same pair of primers used for radioactive fingerprint presentation. Fluorescent *Not*I-*Mse*I MS-AFLP offers a safer method of studying the alterations in DNA methylation, and may be applied to the hybridization of DNA microarrays in the future. Using *Not*I-*Mse*I MS-AFLP, we observed frequent hypomethylation of a satellite DNA repeat sequence in a majority of breast tumors.

Keywords: Methylation-sensitive amplified fragment length polymorphism / DNA methylation analysis / DNA fingerprinting / Fluorescence detection / Cancer-associated satellite DNA hypomethylation

EL 4442

1 Introduction

The cytosine methylation of CpG dinucleotide sequence is the most common form of postreplicative modification of genomic DNA in higher eukaryotes. Using palindromic nature of the sequence, methylation pattern can be transmitted to the daughter strands by the maintenance methylation. Although the underlying mechanisms are poorly understood, somatic *de novo* methylation and demethylation can also occur. Regions containing a high density of CpG dinucleotides are called CpG islands, and they are predominantly associated with coding DNA. In the past years, DNA methylation of CpG islands has been proven to be one important mechanism controlling gene expression. CpG islands of constitutively expressed genes are unmethylated in all the somatic cells and in the germ cells [1]. On the other hand, DNA methylation of CpG islands plays a regulatory role in the tissue-specific gene expression [2]. Imprinting is also tightly associated with alterations in the methylation status of CpG islands [3,4]. DNA hypermethylation seems to be important in the aging process, too [5]. A hypothesis currently accepted is that the subtle alterations in DNA structure may contribute to the changes in chromatin structure, resulting in repression of gene expression [6]. In addition to the normal physiological roles of somatic DNA methylation, aberrations in the mechanisms that ensure its fidelity of perpetuation also play a significant role in pathological processes, one of which is exemplified by cancer development and progression.

All cancers involve a disruption of normal restraints on cell proliferation and survival. Such a disruption may result from genetic and epigenetic alterations. Activation of proto-oncogenes and inactivation of tumor suppressor genes contribute to the uncontrolled cell proliferation, and these result from either mutation in coding sequence, aneuploidy (loss/gain of chromosomes or subchromosomal regions), gene amplification, or transcriptional changes. In all cases, the final consequence is having either more or less of the corresponding functional gene products or altered activity of these products [7]. Hypermethylation of CpG islands in the promoter region is an important mechanism of inactivation through transcriptional controls [8,9]. CpG island hypermethylation has been closely correlated with the transcriptional silencing of certain genes during tumorigenesis. For example, frequent loss of E-cadherin expression in breast and prostate carcinomas was shown to result from hypermethylation of the promoter region [10]. Methylation of *Not*I sites

Correspondence: Fumiichiro Yamamoto, PhD, The Burnham Institute, La Jolla Cancer Research Center, 10901 N. Torrey Pines Rd., La Jolla, CA 92037, USA
E-mail: fyamamoto@burnham.org
Fax: +858-646-3173

Abbreviation: MS-AFLP, methylation-sensitive amplified fragment length polymorphism

© WILEY-VCH Verlag GmbH, 69451 Weinheim, 2001

0173-0835/01/1006–1946 $17.50+.50/0

at D17S5 on chromosome 17p13.3 was found to be tumor-specific in colon, brain, and kidney tumors [11–13]. Somatic hypermethylation of the CpG island was frequently observed in the receptor gene for endothelin-1 (EDNRB) in prostate cancer [14]. Because endothelin-1 receptor mediates clearance of this potent vasoconstrictor and inhibits secretion, decreased expression of the receptors by hypermethylation may be advantageous for cancer progression *in vivo*. Hypermethylation was also reported for the promoter region of hMLH1, a mutator gene involved in hereditary non-polyposis colorectal cancer (HNPCC) and some sporadic gastrointestinal cancer [15].

Methylation analysis has mostly been conducted on the promoter regions of genes important for growth regulation. However, precise mapping of DNA methylation patterns in CpG islands on a genomic scale is essential for understanding diverse biological processes. We have developed a technique, called *Not*I-*Mse*I methylation-sensitive amplified fragment length polymorphism (MS-AFLP). *Not*I-*Mse*I MS-AFLP is an efficient and sensitive method that permits the genome-wide evaluation of the spectrum of epigenetic alterations (and probably genetic alterations also), as well as the identification and cloning of DNA fragments that exhibit alterations. The technique utilizes the DNA fingerprinting advantages of AFLP [16] and methylation sensitivity of the rare-cutter *Not*I endonuclease, and can be applied to the analysis of complex genome of humans.

2 Materials and methods

2.1 Materials

Genomic DNA from four matched pairs of normal and primary tumor prostate tissues, five matched pairs of normal and primary tumor lung tissues, and 17 matched normal, primary tumor, and metastasized tumor when available, breast tissues, was used in the study. The breast and prostate tissues were obtained from the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. Normal and tumor lung tissue DNA was kindly provided by Dr. Yoshinori Murakami (Oncogene Division, NCCRI, Japan). Genomic DNA from human mammary carcinoma cell lines (CAL-51, MCF-7, Hs 578T, MDA-MB468, and BT-20) was also used. Restriction endonuclease *Not*I and T_4 DNA ligase were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Restriction endonuclease *Mse*I and T_4 polynucleotide kinase were obtained from NE Biolabs (Beverly, MA, USA). AmpliTaq DNA polymerase and dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit were purchased from Perkin-Elmer (Foster City, CA, USA). Easytide ^{32}P-γ adenosine 5'-triphosphate (^{32}P-γATP) and ^{32}P-α deoxycytidine 5'-triphosphate (^{32}P-αdCTP) were purchased from NEN-Life Science Products (Boston, MA, USA). pT-Adv vector was from Clontech (Palo Alto, CA, USA), and frozen competent XL1-Blue strain of *Escherichia coli* bacteria and PrimeIt II kit were from Stratagene (La Jolla, CA, USA). ULTRAhyb hybridization solution was obtained from Ambion (Austin, TX, USA). Oligonucleotides were custom-synthesized at Genset (La Jolla, CA, USA) and at Sigma-Genosys (The Woodlands, TX, USA). *Not*I and *Mse*I adaptors were equimolar mixtures of two oligonucleotides. The nucleotide sequences of the two oligonucleotides for the *Mse*I adaptor were 5'-GACGATGAGTCCTGAG and 5'-TACTCAGGACTCAT, and these sequences were identical to those used by Vos *et al.* [16]. The nucleotide sequences of the oligonucleotides for the *Not*I adaptor were 5'-CTCGTAGACTGCGTACC and 5'-GGCCGGTACGCAGTCTAC, and these sequences were modified from their *Eco*RI adaptor sequences. These pairs of oligos were mixed, heated at 65°C for 15 min, and kept at 37°C overnight for annealing before use. Primers used for PCR amplification were *Not*I+N (5'-GACTGCGTACCGGCCGC+N), *Mse*I+N (5'-GATGAGTCCTGAGTAA+N), and *Mse*I+CN (5'-GATGAGTCCTGAGTAA+CN) adaptor primers. The N denotes any one of G, A, T, and C nucleotides. *Not*I+G adaptor primer labeled at the 5'-end with TAMRA fluorescent dye was custom synthesized at Genset.

2.2 *Not*I-*Mse*I MS-AFLP method

Experimental protocol was modified from the original AFLP protocol [16]. Five hundred nanograms of genomic DNA were digested overnight at 37°C in 25 μL of 1 × *Not*I digestion buffer with 5 units of *Not*I and 2 units of *Mse*I. Then 17 μL of 1 mM Tris-HCl, 1 mM EDTA buffer (1–1TE), 5 μL of 10 × T_4 DNA ligase buffer, 1.25 μL each of 5 pmol/μL *Not*I and 50 pmol/μL *Mse*I adaptors, and 1 unit of T_4 DNA ligase were added, and the DNA was incubated overnight at 16°C. The ligated DNA was incubated at 37°C for 2–6 h, heat-denatured at 70°C for 20 min to inactivate the enzymes, and diluted to adjust the concentration to 1 ng/μL. For the radioactive *Not*I-*Mse*I MS-AFLP experiments, one of the primers was radiolabeled at the 5'-end using T_4 polynucleotide kinase and ^{32}P-γATP. *Not*I primer was radiolabeled in all the experiments except one where *Mse*I primer was labeled. Six nanograms of *Not*I primer and 30 ng of *Mse*I primer were used for PCR with adaptor-ligated genomic DNA template in 20 μL of 1 × AmpliTaq DNA polymerase buffer, 0.4 mM dNTP, with 1 unit of AmpliTaq DNA polymerase. Various amounts of adaptor-ligated DNA (16 pg, 80 pg, 400 pg, 2 ng) were used in the experiments with lung DNA. Five ng of adaptor-ligated DNA was used in the breast and prostate

experiments. Two-hundred μL tubes were used and placed in a 96-well-type DNA Thermal Cycler. PCR protocol was the following: 72°C for 30 s, 94°C for 30 s, 36 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min, followed by incubation at 72°C for 10 min. The samples were kept at 10°C until the reaction was terminated by the addition of 25 μL of loading dye solution. After 3 min of heat denaturing followed by a quick chill on ice, an aliquot (4 μL) from each reaction was loaded onto a SEQUAGEL-6 denaturing gel. After electrophoresis, the gel was dried and then exposed to an X-ray film. For the fluorescent MS-AFLP experiments, 30 ng each of fluorescent *Not*I primer and unlabeled *Mse*I primer and 10 ng of template DNA were used in 20 μL of 1xAmpliTaq DNA polymerase buffer, 0.4 mM dNTP, with 1 unit of AmpliTaq DNA polymerase. The same PCR protocol described above was used except that the cycle number was decreased to 31. PCR products were added with 3 μL of 3 M sodium acetate (pH 5.2) and 75 μL of 95% ethanol, and then precipitated. After being washed with 70% ethanol and air dried, the pellets were resuspended in 3 μL of dye containing 5:1 ratio of formamide: 25 mM EDTA (pH 8.0) with blue dextran. Half the samples were then loaded onto an ABI Prism 377 automatic DNA Sequencer and electrophoresed according to the manufacturer's protocols.

2.3 PCR amplification, cloning, and DNA sequencing

DNA was eluted from the excised gel fragment and used as a template for PCR amplification. The same primer pair used for the MS-AFLP was used. Amplified DNA was agarose-gel-electrophoresed, gel-purified, and ligated by the T-A cloning method with pT-Adv vector. Ligated DNA was then used to transform the XL1-Blue strain of *E. coli* bacteria. After DNA transformation, plasmid DNA was prepared from several independent clones and the nucleotide sequences of the inserts were determined using dRhodamine Dye Terminator Cycle Sequencing Ready Reaction kit. Homology search was performed using the BLAST program.

2.4 Southern hybridization

One microgram of genomic DNA from MCF-7, MDA-MB468, and Hs 578T cells was digested with 18 units of *Not*I or *Hpa*II overnight, electrophoresed through a 1.5% agarose gel, and Southern transferred onto a nylon membrane. A radiolabeled probe was prepared, from the amplified DNA fragment from a cloned insert, by the random primer method using ^{32}P-αdCTP and PrimeIt II kit. The membrane filter was hybridized with the probe in the ULTRAhyb hybridization solution overnight at 42°C, washed, and then exposed to an X-ray film. The same probe was also used to hybridize the electrotransfer blot of the MS-AFLP DNA fingerprints.

3 Results

3.1 Theoretical basis of *Not*I-*Mse*I MS-AFLP

*Not*I-*Mse*I MS-AFLP was developed to analyze the methylation status of cytosine residues of the CpG dinucleotides at the *Not*I landmark sites (G**C**GGC**C**GC) scattered in the genome. It was, therefore, not intended to scan all the genes for methylation alterations, and not to scan all the cytosine residues in the entire genome. We modified the AFLP method to use methylation-sensitive *Not*I restriction enzyme in place of *Eco*RI. Unlike hexanucleotide *Eco*RI recognition sites, *Not*I sites are octanucleotides. The human genome is 49% G+C, but the CpG dinucleotide is fivefold rarer than the expected G+C content [17]. As a result, sequences recognized by the *Not*I enzyme are rare. The total number of *Not*I sites in human genome is calculated to be around 10^5. Considering that there are only 25 000–40 000 genes in the human genome [18,19], those 10^5 sites, in our opinion, provide decent coverage of the genome even if only a small fraction of CpG islands contain *Not*I site(s). In certain tissues, approximately 5000 different *Not*I sites are unmethylated and cleavable [20]. This value is much smaller than the estimated 8×10^5 cleavable *Eco*RI sites. Therefore, only one additional nucleotide per primer is sufficient in the *Not*I system to obtain meaningful banding patterns of AFLP fingerprints of the human genome, differing from the *Eco*RI system where at least three additional nucleotides are necessary for each primer. Accordingly, most of the *Not*I fragments (*Not*I-*Mse*I and *Not*I-*Not*I) can be revealed by just 16 reactions (theoretically $5000 \times 2/16=625$ bands per reaction). When two, rather than one, additional nucleotides are added at one of the primers, the number of bands per reaction decreases to 166. Therefore, the alterations in the methylation status of *Not*I sites can be easily distinguished by the changes in band intensity in DNA fingerprints. The *Not*I-*Mse*I MS-AFLP method is schematically depicted in Fig. 1.

3.2 Reproducibility of the *Not*I-*Mse*I MS-AFLP fingerprinting

We examined the reproducibility of *Not*I-*Mse*I MS-AFLP. After digestion with *Not*I and *Mse*I, human genomic DNA was ligated with adaptors and used as a template for MS-AFLP DNA fingerprint presentation. In order to determine the amount of template necessary to obtain reproducible results, titration experiments were performed. ^{32}P-radio-

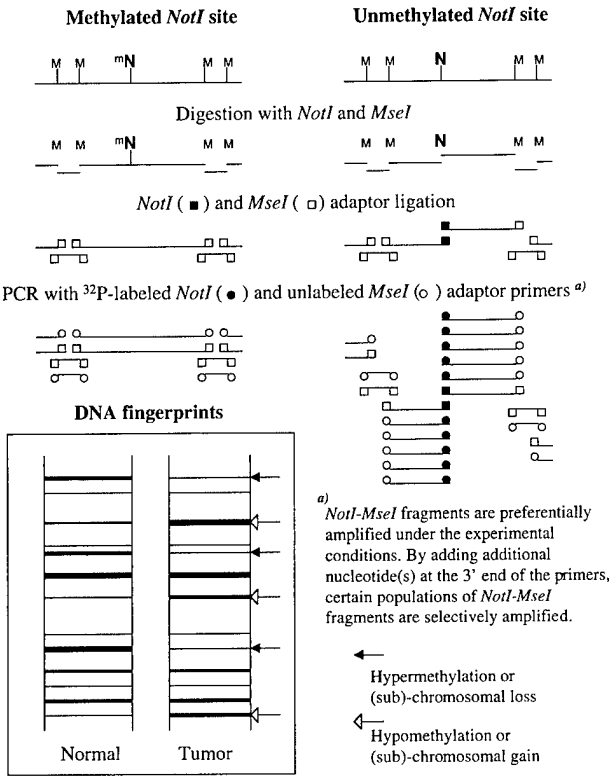

Figure 1. Schematic representation of the *NotI*-*MseI* MS-AFLP method. DNA is digested with *NotI* and *MseI*. All the *MseI* sites are cleaved and *MseI* ends are ligated with *MseI* adaptors. Unmethylated *NotI* sites are cleaved by *NotI* and *NotI* ends are ligated with *NotI* adaptors whereas methylated *NotI* sites are resistant to cleavage, and no adaptors are ligated. *NotI*-*MseI* DNA fragments are preferentially amplified over *MseI*-*MseI* DNA fragments under the experimental condition. By using primers with additional nucleotide(s) at the 3'-end for PCR, only selected populations of DNA fragments are amplified, reducing the number of bands. DNA fingerprints of normal and tumor DNA from an individual is also schematically shown in an insert. The decreased and increased band intensity of MS-AFLP fingerprints may result from hypermethylation (or subchromosomal loss) and hypomethylation (or subchromosomal gain), respectively.

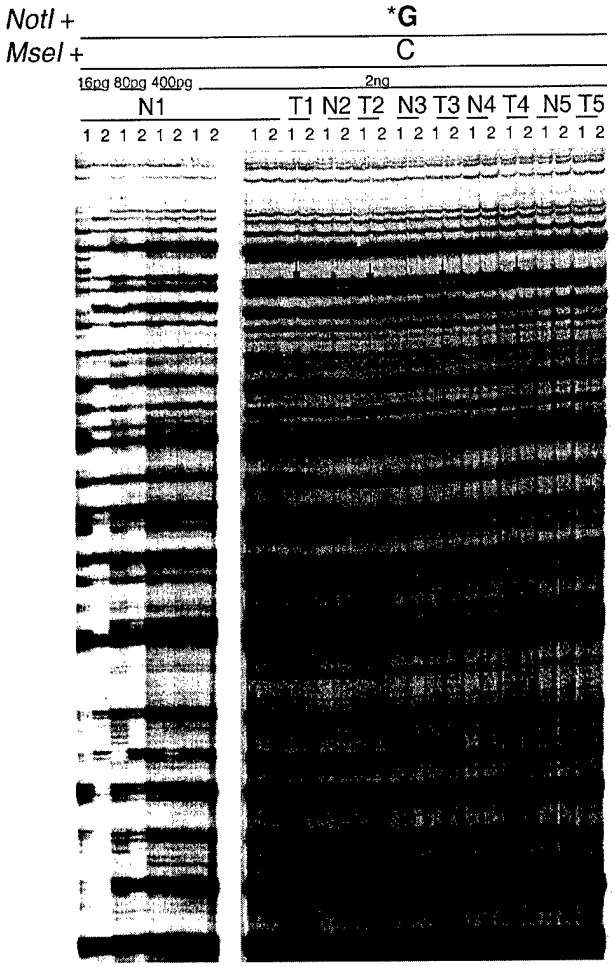

Figure 2. *NotI*-*MseI* MS-AFLP fingerprints of lung cancer. Results from the *NotI*-*MseI* MS-AFLP experiments with one pair of primers (*NotI*+G and *MseI*+C) are shown. Left: different amounts of adaptor ligated template DNA were used for the *NotI*-*MseI* MS-AFLP to determine the minimal amount of DNA required for obtaining reproducible results. Reactions were duplicated and the results are shown with 1 and 2. Right: five pairs of matched normal and tumor lung tissue DNA was analyzed by the *NotI*-*MseI* MS-AFLP. N and T denote normal and tumor tissue DNA, respectively. The arrows indicate the bands that exhibited consistent alterations in band intensity.

labeled *NotI*+G primer was used in combination with unlabeled *MseI*+C primer. Reactions were duplicated in two different tubes, and were electrophoresed in parallel. Adaptor ligated normal lung DNA (N1) was serially diluted, and the same 5 μL containing different amounts of DNA was used for the MS-AFLP in 20 μL reactions. Reaction products were analyzed through denaturing gel electrophoresis. Results are shown in the left panel of Fig. 2. When 16 pg or 80 pg of template DNA was used, banding patterns varied significantly between the duplicate reactions. When the amount of template was increased to 400 pg, banding patterns became similar except for several sporadic changes in band intensity. When 2 ng of template DNA was used, two separate reactions using the same adaptor-ligated DNA produced essentially identical banding patterns. Banding patterns were almost identical among reactions using 2, 5, and 10 ng of DNA template (data not shown). We concluded that at least 2 ng of human genomic DNA (approximately 300 cell equivalents = 600 genome equivalents) was needed to obtain reproducible results.

DNA fingerprints, produced with five pairs of normal (N) and tumor (T) genomic DNA from lung cancer patients, were then examined. The same *Not*I+G and *Mse*I+C primers were used. All the reactions were duplicated. Results are shown in the right column of Fig. 2. Two separate reactions using the same DNA template again produced essentially identical banding patterns. Cases 1 through 4 showed several somatic alterations, whereas few alterations were observed in case 5. Arrows indicate the possible tumor-associated alterations.

3.3 Combinatorial MS-AFLP using adaptor primers with additional nucleotides

In the above experiments, only one pair of primer combination was used. In the next experiment, we used all 16 combinations (4×4) of adaptor primers with one additional nucleotide each at their 3'-ends. Five nanograms of adaptor-ligated DNA from normal breast (N) and metastatic breast carcinoma (M) from the same individual was used as templates. ^{32}P-radiolabeled *Not*I+N primers were used in combination with unlabeled *Mse*I+N primers. The N denotes any one of G, A, T, and C nucleotides. Results are shown in Fig. 3. The combinations of primers used are indicated on the top of the gel. Although some combinations gave better banding patterns than others, basically all the primer combinations worked. One reaction, *Not*I+T and *Mse*I+A reaction with metastasized DNA, failed to give a good quality fingerprint from unknown cause(s). Since the same pair of primers could successfully produce a good quality result with normal DNA from the same patient, this failure does not seem to be a molecular issue.

We estimated the total numbers of bands exhibited by the 16 *Not*I-*Mse*I MS-AFLP fingerprints to be 3200 (200×16). This value was lower than the expected total number 10 000 (5000×2). Possible reasons for this discrepancy include: (i) some bands were represented by more than one DNA fragment, (ii) some fragments with larger sizes or complicated sequences were not amplified efficiently, and (iii) some amplified fragments were too small and ran out of the gel. Nevertheless, the number of different bands obtained seemed to be sufficient for a near saturation scanning of the genome. This number may be doubled to 6400 by the use of two additional nucleotides at the 3' end of one of the primers as will be discussed below.

Multiple somatic fingerprint alterations were observed in this experiment alone, and several examples are indicated with up and down arrowheads in the figure. This demonstrates the potential of the *Not*I-*Mse*I MS-AFLP technique that generated a large number of potentially interesting somatic epigenetic alterations in breast cancer

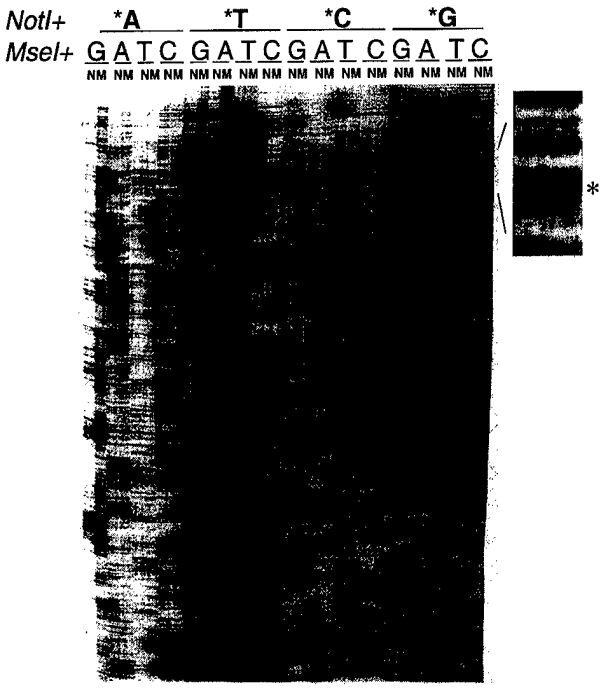

Figure 3. The *Not*I-*Mse*I MS-AFLP fingerprints obtained using all the 16 combinations of primers with one additional nucleotide each. DNA from normal breast (N) and metastatic breast carcinoma (M) from a patient was used for *Not*I-*Mse*I MS-AFLP using 16 combinations of primer pairs with one additional nucleotide at the 3'-end. The added nucleotide residues are shown above the lanes. Examples of prominent bands showing increases or decreases in band intensity in the tumor are indicated by up and down arrowheads, respectively. The position of the band that has been further analyzed is marked with an asterisk in an insert.

from this single experiment. The asterisk indicates the alteration found hypomethylated in a majority of breast tumors, which will be discussed later. An additional property of the technique is that the dried gels can be stored at room temperature for months and even years, and used later to isolate the relevant bands. At the same time, the experiment illustrates another important property of the technique, its ability to obtain a panoramic view of the alterations undergone by the tumor cells.

We next compared the results obtained from *Not*I-*Mse*I MS-AFLP using *Mse*I primer with an additional nucleotide and those obtained using *Mse*I primers with two additional nucleotides. DNA from four sets of matched normal and tumor prostate tissues was used in the experiment. ^{32}P-radiolabeled *Not*I+G primer was used in combination with unlabeled *Mse*I+C for all four sets of DNA, and with unlabeled *Mse*I+CG, *Mse*I+CA, *Mse*I+CT, or *Mse*I+CC for

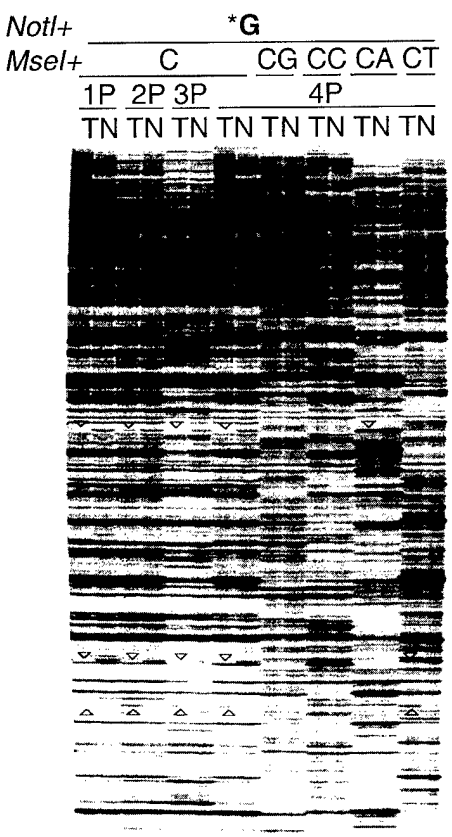

Figure 4. Comparison between fingerprints obtained from the 4×4 format with those from the 4×16 format. Normal (N) and tumor (T) DNA from four prostate cancer patients (1P-4P) was used to compare the results from the 4×4 format and those from the 4×16 format (two additional nucleotides were added to the *Mse*I primers). Three bands showing intensity changes in four prostate samples are indicated by open arrowheads. The adaptor-ligated DNA from case 3P was prepared separately from other samples. That is why the overall pattern of 3P fingerprint was somewhat different from the other three fingerprints. Surprisingly, however, the consistent alterations observed in the other fingerprints were also found in 3P fingerprint, suggesting that the alterations can be identified so far as both normal and tumor samples are processed in parallel.

the fourth set of DNA. Results are shown in Fig. 4. The results demonstrate that many bands in the *Mse*I+C fingerprint were in fact composed of several comigrating bands. Therefore, the use of two additional nucleotides in the primers can be exploited to increase the total number of DNA fragments that can be analyzed and to increase the specificity of identification of sequences with alterations. The fact that the consistent alterations observed in four cases of prostate tumors were also identified in one of the four primer combinations, clearly demonstrated that those observed differences in band intensity were not an artifact but rather genuine sequence-dependent alterations.

3.4 Preferential amplification of *Not*I-*Mse*I fragments over *Mse*I-*Mse*I fragments in *Not*I-*Mse*I MS-AFLP

In addition to the advantage that a small amount of DNA template is sufficient, the PCR-based MS-AFLP may also offer a relatively easy cloning method of DNA fragments from fingerprint bands. This is true, however, only when *Not*I-*Mse*I (and *Not*I-*Not*I) fragments are preferentially amplified. In the above experiments, we used ^{32}P-radiolabeled *Not*I adaptor primers. *Mse*I endonuclease cleaves at the TTAA sequence, and this tetranucleotide sequence is much more abundant in the genome than the octanucleotide *Not*I sequence. Therefore, it was possible that in addition to the radiolabeled *Not*I-*Mse*I and *Not*I-*Not*I fragments that sensitized the X-ray film, there might have been thousand times more of the unlabeled *Mse*I-*Mse*I fragments in the background. In order to examine this possibility, we performed MS-AFLP experiments using the same primer combination but using ^{32}P-radiolabeled *Mse*I adaptor primers and compared with the results from the MS-AFLP experiments with ^{32}P-radiolabeled *Not*I adaptor primers. Results are shown in Fig. 5. The two leftmost and the two rightmost lanes show the two pairs of MS-AFLP fingerprints using the same combinations of primers but labeled at different primers. The two lanes exhibited quite similar, though not identical, results that implicated that *Not*I-*Mse*I (and *Not*I-*Not*I also?) fragments were selectively amplified over *Mse*I-*Mse*I fragments under the PCR conditions employed.

3.5 Fluorescent *Not*I-*Mse*I MS-AFLP

One advantage of using radioactive tags for *Not*I-*Mse*I MS-AFLP is that it allows direct cloning of DNA fragments after the identification of alterations. However, use of fluorescent tags offers a different kind of advantage. It is safer, and this safe feature is especially important in clinical settings. Simultaneous usage of multiple dyes with different absorption and emission ranges is possible. Therefore, we investigated the conditions for labeling the MS-AFLP products with a fluorescent primer, and examined the sensitivity of the detection. Highly reproducible fingerprint profiles were obtained using the protocol detailed in Section 2. Results from the fluorescent MS-AFLP experiments using a pair of normal and tumor DNA from a breast cancer patient are shown in Fig. 6. Almost identical peak profiles were obtained among the triplicate normal (N_1, N_2, and N_3) and duplicate tumor (T_1 and T_2)

Figure 5. Comparison of the *Not*I-*Mse*I MS-AFLP fingerprints produced from reactions using ^{32}P-radiolabeled *Not*I adaptor primers and ^{32}P-radiolabeled *Mse*I adaptor primers. Results from *Not*I-*Mse*I MS-AFLP experiments using the same primer combinations but labeled at either *Not*I or *Mse*I primers are shown in the two leftmost and two rightmost lanes. PCR products were analyzed by parallel electrophoresis.

reactions. A few examples of the increased and decreased peak intensity alterations observed in the tumor electropherograms are indicated by up and down arrowheads respectively.

3.6 Widespread satellite DNA hypomethylation in breast tumors

We excised the gel fragment containing the band in one of the *Not*I-*Mse*I MS-AFLP fingerprints, which exhibited a conspicuous increase in band intensity in the DNA fingerprint of the metastasized breast tumor as shown by the asterisk in Fig. 3. We eluted DNA and amplified the fragment by PCR using *Not*I+G and *Mse*I+C primers, the same pair of primers used in the fingerprinting experiments. The amplified DNA fragment was cloned into pT-Adv vector, and the nucleotide sequence was determined. A homology search revealed that the sequence had significant homology with tandemly repeated sequences from pericentromeres of chromosomes 9, 13, 14, and 21 (U59100: 5e-69 probability and Y10572: 7e-65). Genomic DNA from additional breast cancer cases was next examined for this alteration by the *Not*I-*Mse*I MS-AFLP. Stronger band intensity in tumor was observed in a majority of breast cancer patients (12 out of 17 cases). Results from one such experiment are shown in Fig. 7A. Genomic DNA from breast cancer cell lines was also examined for the alteration. Results are shown in the left panel of Fig. 7B. A strong signal was observed with DNA from Hs 578T cell line and a weak signal was observed with DNA from MDA-MB468 and BT-20 cell lines. DNA from these fingerprints was electrotransferred onto a nylon membrane and hybridized with the radiolabeled, cloned satellite DNA fragment probe. Results are shown in the right panel of Fig. 7B. The identical banding pattern was obtained, confirming the identity of the band. Weak signal was detected with DNA from CAL-51 cells by this method with higher sensitivity. No signal was observed with DNA from MCF-7.

We then performed the Southern hybridization experiment. Genomic DNA from MCF-7, MDA-MB468, and Hs 578T cells was digested with *Not*I or *Hpa*II, gel electrophoresed, and Southern transferred. Results of electrophoresis and hybridization are shown in the left and the right panels of Fig. 7C. The relative hybridization signal to DNA amount seems to be stronger with MDA-MB468 DNA than those with MCF-7 and Hs 578T DNA. This may be due to a difference in the degree of homology to the nucleotide sequence of the repeat fragment used as a probe or a difference in the number of repeats. Nonetheless, differential susceptibility to those methylation sensitive restriction enzymes seems obvious.

4 Discussion

Precise mapping of DNA methylation patterns in CpG islands is essential for understanding diverse physiological and pathological biological processes. Several techniques have been developed to determine DNA methylation alterations of specific genes once their sequences are identified [21–24]. In addition to the established methods of digestion with methylation-sensitive restriction enzyme(s) followed by Southern hybridization or PCR, methylation-specific PCR (MSP) has been developed as a novel PCR assay for determining the methylation status of CpG islands [22]. Using this technique, hypermethylation associated with transcriptional inactivation was reported in the promoter regions of four important tumor suppressor genes in human cancer (p16 [25], p15 [26], E-cadherin [10], and von Hippel-Lindau [27]). High-sensitivity methylation mapping is

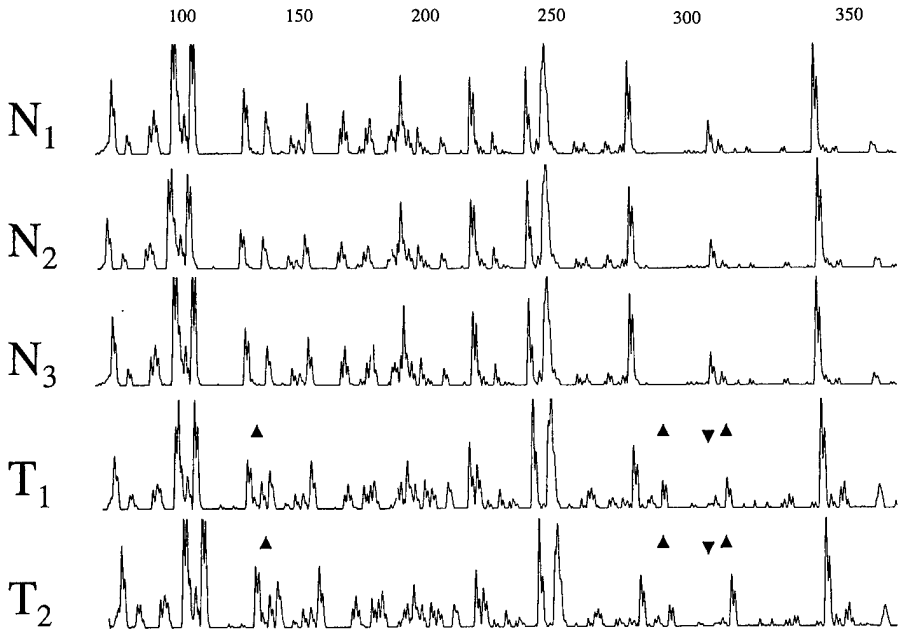

Figure 6. Fluorescent *Not*I-*Mse*I MS-AFLP. Fluorescently labeled *Not*I+G primer and unlabeled *Mse*I+C primer were used for *Not*I-*Mse*I MS-AFLP, and the results were analyzed using an automatic DNA sequencer. Results from triplicate normal (N_1, N_2, and N_3) and duplicate tumor (T_1 and T_2) fluorescent *Not*I-*Mse*I MS-AFLP reactions are shown. Electropherograms were obtained by detecting fluorescent signals derived from TAMRA dye (corresponding to the T-reaction of the DNA sequencing reactions). A few examples of increased and decreased peaks in the tumor are indicated by up and down arrowheads, respectively.

possible by the sodium bisulfite modification-DNA sequencing method [21]. The methylation-sensitive single nucleotide primer extension (Ms-SNuPE) method is based on bisulfite treatment of DNA followed by single nucleotide primer extension [23]. This method has been successfully used to measure methylation of the 5'-CpG islands of c-abl and p15 in chronic myelogenous leukemia (CML) patients during progression [28] and methylation of exon 2 shared by the p16 and p14 genes in prostate cancer [29]. Techniques are also available for determining the degree of methylation in bulk DNA [30]. However, there are few techniques that generate a comprehensive methylation profile of human genomic DNA. Three gel electrophoresis-based scanning techniques are currently available for DNA methylation analysis: methylation-sensitive restriction fingerprinting (MSRF) [31], restriction landmark genomic scanning for methylation (RLGS-M) [32], and amplification of restriction polymorphism [33].

The MSRF method employs the principle of the AP-PCR approach. After digesting genomic DNA with *Mse*I alone or *Mse*I and methylation sensitive *Bst*UI, the DNA is PCR-amplified using a specially designed pair of primers with short arbitrary 10-mer sequences attached to the flanking *Bst*UI recognition sequence (CGCG). The PCR products are electrophoresed and banding patterns compared between the *Mse*I digestion alone and the *Mse*I/*Bst*UI double digestion. Although this approach has successfully identified two breast carcinoma-specific hypermethylation fragments (HBC-1 and 2), scanning an entire genome in an organized manner remains an impossible task when using this method with arbitrary primer sequences. RLGS-M, another scanning technique, allows genomic scanning for changes in methylation at *Not*I sites. Because genomic DNA is directly end-labeled in RLGS-M, different from PCR-based techniques, the ratio of all the fragments remains unchanged. Moreover, the entire fingerprints can be obtained from one reaction displayed on a two-dimensional gel. However, two-dimensional gel electrophoresis-based RLGS-M is complicated, and is not suitable for simultaneous analyses of multiple specimens although some success has been reported using a specially designed apparatus [34, 35]. Other shortcomings are that RLGS-M requires microgram quantities of good quality DNA to visualize individual genomic DNA fragment spots, and cloning of the DNA fragments are extremely difficult. Similar to *Not*I-*Mse*I MS-AFLP, amplification of restriction polymorphism method utilizes the AFLP technique and a methylation-sensitive restriction enzyme. Different combinations of enzymes (*Eco*RI and *Hpa*II/*Msp*I) are used, however.

The *Not*I-*Mse*I MS-AFLP method is superior to other scanning techniques for DNA methylation alterations in several aspects. MSRF is similar to MS-AFLP in that the alterations are detected in one-dimensional denaturing sequencing gels, and the amplified products are relatively small DNA fragments representing anonymous genomic sequences. The advantage of our method is its superior reproducibility. The larger number of bands are amplified and the higher ratio of band/background noise is obtained (see Figs. 2–5). Another advantage of the *Not*I-*Mse*I MS-AFLP approach is that it may be more benefited from the Human Genome Project effort since the posi-

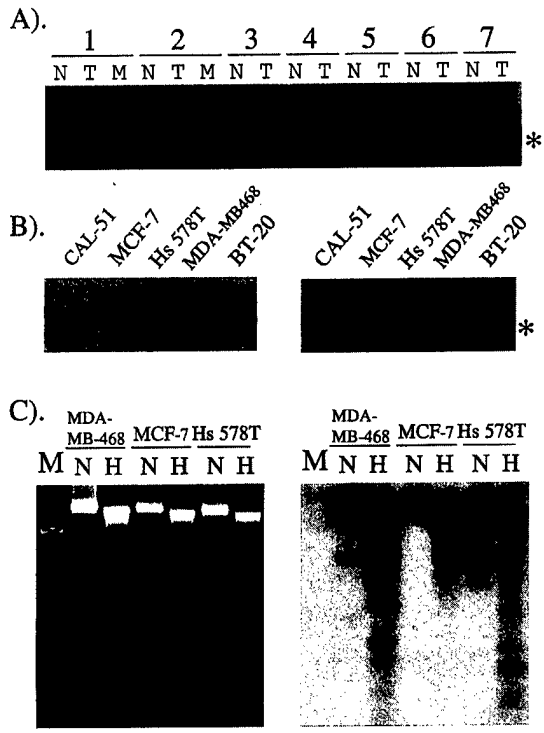

Figure 7. Satellite DNA hypomethylation in breast cancer. (A) The area that surrounds the satellite DNA band from the *Not*I-*Mse*I MS-AFLP fingerprints is shown. Two sets of normal, primary, and metastasized tumor tissues and five pairs of normal and primary tumor tissues from breast cancer patients were analyzed for the alteration by the *Not*I-*Mse*I MS-AFLP. The position of the satellite DNA bands is marked with an asterisk. (B) The intensity of the band was also examined with breast cancer cell line DNA. Results from the MS-AFLP fingerprinting and hybridization experiments are shown in the left and the right panel, respectively. (C) The Southern hybridization experiment was performed using the radiolabeled probe prepared from the cloned satellite DNA fragment. DNA from three breast cancer cell lines was digested with either *Not*I (N) or *Hpa*II (H). The left panel shows the results of electrophoresis, and the right panel shows the results of hybridization. The symbol (M) denotes 1 kbp and 100 bp DNA ladder mix markers.

tions of the *Not*I sites in the genome will be easily identified compared with more abundant *Bst*UI sites used in the MSRF approach. Therefore, with a defined *Not*I map, *Not*I-*Mse*I MS-AFLP is advantageous for the systematic comparative screening of different situations. Compared with RLGS-M, MS-AFLP is clearly superior in several aspects. First, MS-AFLP requires much less template DNA. We usually use 5 ng of DNA for one MS-AFLP experiment with one pair of primers. If 16 reactions are performed by the 4×4 format, a total of 80 ng of DNA is needed. Preamplification of the adaptor-ligated DNA fragments with universal primers without additional nucleotide at the 3'-end before fingerprint presentation may reduce even more the total amount of template (data not shown). Moreover, simultaneous analysis of multiple samples is easily performed by parallel electrophoresis, which also facilitates the detection of consistent differences in band intensity. More importantly, in contrast to RLGS-M, MS-AFLP permits the single-step cloning of the fingerprint bands from the gel by PCR with the adaptor primers.

The amplification of restriction polymorphism method is advantageous in its easy confirmation of DNA methylation alterations by side-by-side examination of the *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I DNA fingerprints since both *Hpa*II and *Msp*I recognize the same CCGG sequence but differ in their sensitivity to the methylated sequence. This method analyzes the methylation of the nearest *Hpa*II/*Msp*I sites of *Eco*RI sites whereas *Not*I-*Mse*I MS-AFLP analyzes the methylation of *Not*I sites. This is an important issue since *Not*I sites are frequently found in the CpG islands associated with gene promoters whereas *Eco*RI sites are not. Additionally, the number of *Eco*RI-*Hpa*II/*Msp*I fragments is too large in the human genome, and the conditions to obtain reproducible results are tricky. Although this may be overcome by employing a two-step amplification, preamplification followed by amplification using more selective nucleotides, the use has been limited to the genomic analysis of organisms with relatively small genome sizes [33, 36].

In addition to the radioactive *Not*I-*Mse*I MS-AFLP, we have also attempted the fluorescent *Not*I-*Mse*I MS-AFLP. Since the conditions of PCR amplification, gel electrophoresis, and the method of detection were different, it seemed to be rather difficult, if not impossible, to correlate all the individual bands in radioactive MS-AFLP fingerprints and the individual peaks in fluorescent MS-AFLP electropherograms. Nonetheless, there was a clear positive correlation between the number of alterations in radioactive MS-AFLP and the number of alterations in fluorescent MS-AFLP (data not shown), suggesting that fluorescent MS-AFLP is also useful in detecting the differences. Considering the recent success of detecting DNA methylation alterations by hybridizing DNA miniarrays on nylon membranes with radiolabeled probes [37], it may soon become possible to perform the CGH-type hybridization experiments by probing DNA microarrays on glass slides with differentially labeled fluorescent *Not*I-*Mse*I MS-AFLP products. The determination of the identity of alterations may be greatly facilitated that way. In conclusion, *Not*I-*Mse*I MS-AFLP provides another method of genome-wide DNA methylation analysis in both research and clinical settings.

Using the *Not*I-*Mse*I MS-AFLP method, we have analyzed DNA methylation alterations associated with tumorigenesis. This has resulted in the identification of dozens of

bands that exhibited alterations in band intensity. Among those, one band was further characterized to have derived from tandemly repeated satellite DNA from pericentromeric regions of chromosomes. Methylation alterations in the repetitive sequences are easy to detect since the difference in band intensity is conspicuous due to high copy number. However, this does not imply that a large number of differences in the banding pattern are caused by differentially methylated repetitive sequences since most, if not all, repetitive sequences are represented by single bands.

The centromere is the constricted region of a chromosome at which the chromosome is attached to microtubules, and therefore is essential for chromosomal segregation during mitosis. The pericentromeres, the regions flanking the centromere are often rich in short repeated satellite DNA sequences and also contain a considerable amount of constitutive heterochromatin. A deficiency in genomic methylation at satellite DNA sequences on either side of the centromeres has been reported in the ICF syndrome (immunodeficiency, centromeric region instability, facial anomalies) [38–40]. Mutations have recently been found in the coding sequence of Dnmt3b DNA methyltransferase gene in the patients with this hereditary disorder [41–43]. In addition to this syndrome, hypomethylation in the satellite DNA sequences of the pericentromeric regions of chromosomes has also been reported in various cancers. These include melanoma cell lines [44], immunodeficiency virus-related non-Hodgkin's lymphoma [45], breast adenocarcinomas [46], ovarian cancers [47], and Wilms tumors [48]. We found that the *Not*I site in the tandem repeat sequences from pericentromeres of chromosomes 9, 13, 14, and 21 were hypomethylated in a majority of breast tumors. Since the hypomethylation of this *Not*I site has been reported in neuroblastomas [49] and HBV-integrated hepatocellular carcinomas [50], this satellite hypomethylation may be important in carcinogenesis, predisposing cells to structural and numerical chromosomal aberrations through the altered interaction with centromere binding proteins [51].

We thank Fumiya Yamamoto for editing the manuscript and preparing the figures. We also thank Dianne Foster and Tomoko Maruyama for technical assistance. This work was supported by grants from the California Breast Cancer Research Program (4JB144) and from the Department of Defense Breast Cancer Research Program (DAMD17-99-1-9095) to FY, and a grant from NIH (5R37CA63585) to MP. FY, JLS, and MP were Foreign Research Fellows of the Foundation for Promotion of Cancer Research, Tokyo.

Received November 29, 2000

5 References

[1] Bird, A., Taggart, M., Frommer, M., Miller, O. J., Macleod, D., *Cell* 1985, *40*, 91–99.

[2] Cedar, H., *Cell* 1988, *53*, 3–4.

[3] Constancia, M., Pickard, B., Kelsey, G., Reik, W., *Genome Res.* 1998, *8*, 881–900.

[4] Heard, E., Clerc, P., Avner, P., *Annu. Rev. Genet.* 1997, *31*, 571–610.

[5] Issa, J. P., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E., Baylin, S. B., *Nat. Genet.* 1994, *7*, 536–540.

[6] Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., Wolffe, A. P., *Nat. Genet.* 1998, *19*, 187–191.

[7] Weinberg, R. A., *Ann. N. Y. Acad. Sci.* 1995, *758*, 331–338.

[8] Laird, P. W., Jaenisch, R., *Hum. Mol. Genet.* 1994, *3*, 1487–1495.

[9] Klein, C. B., Costa, M., *Mutat. Res.* 1997, *386*, 163–180.

[10] Graff, J. R., Herman, J. G., Lapidus, R. G., Chopra, H., Xu, R., Jarrard, D. F., Isaacs, W. B., Pitha, P. M., Davidson, N. E., Baylin, S. B., *Cancer Res.* 1995, *55*, 5195–5199.

[11] Makos, M., Nelkin, B. D., Lerman, M. I., Latif, F., Zbar, B., Baylin, S. B., *Proc. Natl. Acad. Sci. USA* 1992, *89*, 1929–1933.

[12] Makos, M., Nelkin, B. D., Reiter, R. E., Gnarra, J. R., Brooks, J., Isaacs, W., Linehan, M., Baylin, S. B., *Cancer Res.* 1993, *53*, 2719–2722.

[13] Makos, M., Nelkin, B. D., Chazin, V. R., Cavenee, W. K., Brodeur, G. M., Baylin, S. B., *Cancer Res.* 1993, *53*, 2715–2718.

[14] Nelson, J. B., Lee, W. H., Nguyen, S. H., Jarrard, D. F., Brooks, J. D., Magnuson, S. R., Opgenorth, T. J., Nelson, W. G., Bova, G. S., *Cancer Res.* 1997, *57*, 35–37.

[15] Kane, M. F., Loda, M., Gaida, G. M., Lipman, J., Mishra, R., Goldman, H., Jessup, J. M., Kolodner, R., *Cancer Res.* 1997, *57*, 808–811.

[16] Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., et al., *Nucleic Acids Res.* 1995, *23*, 4407–4414.

[17] McClelland, M., Ivarie, R., *Nucleic Acids Res.* 1982, *10*, 7865–7877.

[18] Consortium, I. H. G. S., *Nature* 2001, *409*, 860–921.

[19] Venter, J., Adams, MD., Myers, EW., Li, PW., Mural, RJ., Sutton, G.G., *et al.*, *Science* 2001, *291*, 1304–1351.

[20] Yoshikawa, H., de la Monte, S., Nagai, H., Wands, J. R., Matsubara, K., Fujiyama, A., *Genomics* 1996, *31*, 28–35.

[21] Clark, S. J., Harrison, J., Paul, C. L., Frommer, M., *Nucleic Acids Res.* 1994, *22*, 2990–2997.

[22] Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., Baylin, S. B., *Proc. Natl. Acad. Sci. USA* 1996, *93*, 9821–9826.

[23] Gonzalgo, M. L., Jones, P. A., *Nucleic Acids Res.* 1997, *25*, 2529–2531.

[24] Xiong, Z., Laird, P. W., *Nucleic Acids Res.* 1997, *25*, 2532–2534.

[25] Jarrard, D. F., Bova, G. S., Ewing, C. M., Pin, S. S., Nguyen, S. H., Baylin, S. B., Cairns, P., Sidransky, D., Herman, J. G., Isaacs, W. B., *Genes Chromosomes Cancer* 1997, *19*, 90–96.

[26] Herman, J. G., Jen, J., Merlo, A., Baylin, S. B., *Cancer Res.* 1996, *56*, 722–727.

[27] Waber, P. G., Lee, N. K., Nisen, P. D., *Oncogene* 1996, *12*, 365–369.

[28] Nguyen, T. T., Mohrbacher, A. F., Tsai, Y. C., Groffen, J., Heisterkamp, N., Nichols, P. W., Yu, M. C., Lubbert, M., Jones, P. A., *Blood* 2000, *95*, 2990–2992.

[29] Nguyen, T. T., Nguyen, C. T., Gonzales, F. A., Nichols, P. W., Yu, M. C., Jones, P. A., *Prostate* 2000, *43*, 233–242.

[30] Singhal, R. P., Mays-Hoopes, L. L., Eichhorn, G. L., *Mech. Ageing Dev.* 1987, *41*, 199–210.

[31] Huang, T. H., Laux, D. E., Hamlin, B. C., Tran, P., Tran, H., Lubahn, D. B., *Cancer Res.* 1997, *57*, 1030–1034.

[32] Hatada, I., Sugama, T., Mukai, T., *Nucleic Acids Res.* 1993, *21*, 5577–5582.

[33] Reyna-Lopez, G. E., Simpson, J., Ruiz-Herrera, J., *Mol. Gen. Genet.* 1997, *253*, 703–710.

[34] Akama, T. O., Okazaki, Y., Ito, M., Okuizumi, H., Konno, H., Muramatsu, M., Plass, C., Held, W. A., Hayashizaki, Y., *Cancer Res.* 1997, *57*, 3294–3299.

[35] Hayashizaki, Y., Shibata, H., Hirotsune, S., Sugino, H., Okazaki, Y., Sasaki, N., Hirose, K., Imoto, H., Okuizumi, H., Muramatsu, M., *et al., Nat. Genet. 1994, 6, 33–40.*

[36] Xiong, L. Z., Xu, C. G., Saghai Maroof, M. A., Zhang, Q., *Mol. Gen. Genet.* 1999, *261*, 439–446.

[37] Huang, T. H., Perry, M. R., Laux, D. E., *Hum. Mol. Genet.* 1999, *8*, 459–470.

[38] Miniou, P., Jeanpierre, M., Blanquet, V., Sibella, V., Bonneau, D., Herbelin, C., Fischer, A., Niveleau, A., Viegas-Pequignot, E., *Hum. Mol. Genet.* 1994, *3*, 2093–2102.

[39] Smeets, D. F., Moog, U., Weemaes, C. M., Vaes-Peeters, G., Merkx, G. F., Niehof, J. P., Hamers, G., *Hum. Genet.* 1994, *94*, 240–246.

[40] Jeanpierre, M., Turleau, C., Aurias, A., Prieur, M., Ledeist, F., Fischer, A., Viegas-Pequignot, E., *Hum. Mol. Genet.* 1993, *2*, 731–735.

[41] Hansen, R. S., Wijmenga, C., Luo, P., Stanek, A. M., Canfield, T. K., Weemaes, C. M., Gartler, S. M., *Proc. Natl. Acad. Sci. USA* 1999, *96*, 14412–14417.

[42] Okano, M., Bell, D. W., Haber, D. A., Li, E., *Cell* 1999, *99*, 247–257.

[43] Chen, R. Z., Pettersson, U., Beard, C., Jackson-Grusby, L., Jaenisch, R., *Nature* 1998, *395*, 89–93.

[44] Miwa, W., Yashima, K., Sekine, T., Sekiya, T., *Electrophoresis* 1995, *16*, 227–232.

[45] Sawyer, J. R., Swanson, C. M., Koller, M. A., North, P. E., Ross, S. W., *Cancer* 1995, *76*, 1238–1244.

[46] Narayan, A., Ji, W., Zhang, X. Y., Marrogi, A., Graff, J. R., Baylin, S. B., Ehrlich, M., *Int. J. Cancer* 1998, *77*, 833–838.

[47] Qu, G., Dubeau, L., Narayan, A., Yu, M. C., Ehrlich, M., *Mutat. Res.* 1999, *423*, 91–101.

[48] Qu, G. Z., Grundy, P. E., Narayan, A., Ehrlich, M., *Cancer Genet. Cytogenet.* 1999, *109*, 34–39.

[49] Thoraval, D., Asakawa, J., Wimmer, K., Kuick, R., Lamb, B., Richardson, B., Ambros, P., Glover, T., Hanash, S., *Genes Chromosomes Cancer* 1996, *17*, 234–244.

[50] Nagai, H., Baba, M., Konishi, N., Kim, Y. S., Nogami, M., Okumura, K., Emi, M., Matsubara, K., *DNA Res.* 1999, *6*, 219–225.

[51] Kipling, D., Warburton, P. E., *Trends Genet.* 1997, *13*, 141–145.